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C.G.HUFF & F. COULSTON

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THE DEVELOPMENT OF *PLASMODIUM GALLINACEUM* FROM SPOROZOITE TO ERYTHROCYTIC TROPHOZOITE*

CLAY G HUFF AND FREDERICK COULSTON

From the Department of Bacteriology and Parasitology, The University of Chicago

Presented here is a detailed account of the stages in the life cycle of a malarial parasite which develop from sporozoites and which ultimately give rise to the stages parasitizing erythrocytes. This is the first of a series of papers presenting the results of research which was begun in October of 1940 with the view toward elucidation of the fate of the sporozoite in the vertebrate host. In October, 1941 this work was continued under government contract at which time our efforts toward solution of the problem were transferred from *P. cathe-merium* to *P. gallinaceum*. Preliminary reports were made in 1942 and a fairly complete report of the work was made later (1944). In subsequent papers we plan to present similar accounts of other species of parasites, to show the part the early stages can play in chemoprophylactic studies and in problems of natural and acquired immunity. At the outset we should like to caution against the extension of our conclusions to other species, particularly to the human parasites, until studies on such forms have been completed.

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HISTORICAL

A complete historical account of all contributions to our knowledge of the first generations of malarial parasites in the vertebrate host would be of such length as to preclude its inclusion in this paper. Although such an account has yet to be written, some fairly detailed reviews of certain phases of the problem are available (Verney, 1938; Porter and Huff, 1940; Kikuth and Mudrow, 1940). We shall attempt to present here enough of the pertinent history to serve as orientation for the results of the experiments presented below and for related experiments to be presented in some subsequent papers.

The stages of the malarial parasites first to be discovered were the erythrocytic stages which were observed in man by Laveran in 1880 and in birds by Danilewsky (1885). The discovery of the sporogonous cycle in mosquitoes resulted from the ideas of Sir Patrick Manson transmitted to Ross in 1894 and the investigations of Ross (1898) and of Grassi, Bignami and Bastianelli (1899). The details of this cycle which occurs in mosquitoes were so completely and beautifully elucidated by Grassi (1901) in his classic monograph as to need only very minor change today. The gap in our knowledge of the events which occur between the entrance of the sporozoite into the vertebrate host and the infection of the erythrocytes has, however, continued to exist until recent years and, indeed, cannot be said to be completed yet for many species of malarial parasites.

Two theories of the development of sporozoites have been held from the

time of the discovery of the sporogonous development in the mosquito. One of these was the obvious assumption that the sporozoite entered directly into the red blood cell and initiated the parasitemia; the other held that some intermediate development occurred between the injection of the sporozoite into the vertebrate host and the beginning of the attack on the erythrocytes. Grassi (1899) sponsored the latter view because he believed the nuclear differences between sporozoites and the young trophozoites were too great to accord with the theory of the direct entry of sporozoites into erythrocytes. However, the lucid description by Schaudinn (1902) of his observation of the direct entry of the sporozoite into erythrocytes which was accompanied by convincing drawings practically removed the question from the realm of theory and established the direct-entry conception in the minds of most malariologists as an established fact. After 20 years there began a period of accumulation of evidence, much of it of an indirect nature, against the validity of this view. This evidence falls into the 4 categories: (1) the inefficacy of chemoprophylaxis, (2) failure of attempts to repeat Schaudinn's observations, (3) the existence of a period of non-infectivity of the blood and of the infectivity of certain organs following both mosquito bite and injection of sporozoites, and (4) the existence of exoerythrocytic stages in the life cycles of certain species of malarial parasites.

In 1922 the Sargent brothers showed that quinine injected in large doses on the day that canaries were bitten by *Culex* mosquitoes infected with *P. relictum*, did not prevent infection in the bitten birds. Yorke and MacFie (1924) in their studies on *P. vivax* infections in general paretics, observed a similar difference in the efficacy of quinine in

preventing infections induced by blood inoculation on the one hand and those induced by sporozoites on the other. Similar observations by James (1931) led him to discard the prevalent view that sporozoites enter erythrocytes directly and to establish the theory that there exists a tissue stage intermediate between the sporozoite and the erythrocytic stages. The observations of the Sergents, Yorke and Mackie, and James upon the inefficacy of the antimalarial drugs were confirmed and extended to other species of malarial parasites by many others including Swellengrebel and de Buck (1931), Russell and Nono (1932), Kikuth and Giovannola (1932), and Missiroli (1937).

Numerous investigators have tried in vain to observe the direct penetration of sporozoites of various species of malarial parasites into the erythrocytes of susceptible hosts. These include Yorke and MacFie (1924), Yorke (1931), Boyd and Stratman-Thomas (1934), and Raffaele (1934). On basis of the many negative experiments done in this laboratory beginning in 1930 by Huff, by Gingrich and by Stauber which were not published, it is our opinion that many other unpublished attempts to confirm Schaudinn's observations have been made.

It is known that by increasing the number of erythrocytic parasites in the inoculum the incubation period may be decreased in length to the point of its complete disappearance. By contrast, following sporozoite inoculations a certain minimum "negative phase" exists during which the blood is noninfectious when subinoculated to susceptible hosts. (Boyd and Stratman-Thomas, 1934; Raffaele, 1936; 1937; Warren and Coggeshall, 1937; Ciuca *et al.*, 1937; Missiroli, 1937; Kikuth and Mudrow, 1938; Henry, 1939; Boyd and Matthews, 1939). In spite of this period of non-

infectivity of the blood in the incubation period it was shown by Warren and Coggeshall (1937) that the spleen, liver and bone marrow and inoculation site were capable of producing infection when removed during this negative phase and injected into susceptible hosts. Similar results were obtained by Kikuth and Mudrow (1938) and by DeCourt and Schneider (1938) who extended the list of organs thus infected during this negative phase to include lung, kidney, brain, ovary, lymph node and thymus.

Before any stages in the malarial life cycle were discovered which live outside of erythrocytes the theory that an intermediate state might exist between the sporozoite and the trophozoite was, indeed, rather a bold one. In 1930 Huff described a new species of avian malarial parasite, *Plasmodium elongatum*, in which the segmenters "are abundant in haematopoietic tissues." This species was destined to play an important role in introducing a new chapter into malariology. Ben-Harel (1923) was probably the first to study this parasite. In her studies of the tissue localizations of a strain of avian malaria which she called *P. praecox* she observed unusual concentrations of the parasites in the hemopoietic organs. Not only does her figure 1, pl. xv, which depicts an elongate gametocyte, present clear evidence that her strain was a mixture of *elongatum* with the form she called *praecox* but Hartman and Huff were able in 1927 (unpublished work) to obtain the strain upon which she had made her observations and to demonstrate that the species later to be described as *P. elongatum* was actually present in it. Another strain of this species was isolated by Boyd in Georgia in 1924 (see Huff, 1930) and studied by Huff in 1928. Huff and Bloom (1935) made the discovery that this species was capable of

living in all blood and blood-forming cells of the canary, and that the parasites were unpigmented except in hemoglobin-containing cells. Raffaele (1934a) independently observed that a strain of this species isolated in Italy developed preferably in young red cells and that pigment was often missing. Later he (1934b) claimed that development occurred as readily in reticulo-endothelial cells as in erythrocytes, a point at variance with Huff and Bloom's observations and since doubted by Corradetti (1938) and not confirmed by Porter (1942).

Raffaele (1936b) found in sporozoite-induced infections of *P. relictum* a few large, unpigmented schizonts in the organs of the infected birds on the day that erythrocytic parasites appeared in the blood. The existence of such forms in this species has been confirmed by many authors (see Porter and Huff (1940) for a review) and extended to *P. gallinaceum* by James and Tate (1938), to *P. cathemerium* by Kikuth and Mudrow (1937) and to *P. circumflexum* by Coulston (1939) and Manwell and Goldstein (1939ab). The term *exoerythrocytic stage* was applied by James and Tate (1938) to the type of parasite in *P. gallinaceum* which lives outside the erythrocytes. Porter and Huff (1940) called attention to the differences between the type of exoerythrocytic stages which occur in *P. elongatum* and those which are found in infections with *relictum*, *gallinaceum*, *cathemerium*, and *circumflexum*. Porter (1942) applied the term "elongatum-type" to those which appear as morphologically identical stages in a complete erythrocytic series of cells and less frequently in other types of cells and the term "gallinaceum-type" to those which possess a schizogonic series morphologically different from the erythrocytic series and which live predominantly in the lymphoid-macro-

phage system of cells and endothelium. Strains of *cathemerium* and *relictum* which have lost their exoerythrocytic stages are known to regain them after passage of the strains through mosquitoes. (Raffaele, 1936; Kikuth and Mudrow, 1938b; Porter, 1942). Aside from a few unconfirmed reports no evidence has been found for the existence of exoerythrocytic stages in human or simian malarial parasites or in many species of avian malarial parasites (reviewed by Porter and Huff, 1940). Thompson and Huff (1944b) found no evidence of their presence in animals naturally infected with two species of saurian malarial parasites but they (1944a) found another species, *P. mexicanum*, infecting lizards which possesses both elongatum and gallinaceum types of exoerythrocytic stages.

While the discovery of stages in the life cycles of several species of malarial parasites capable of living outside erythrocytes made more plausible a belief in the possible existence of intermediate stages in the development of sporozoites, it was not proof that such stages exist. The demonstrations that exoerythrocytic stages could arise from a single, isolated erythrocytic stage (Coulston and Manwell, 1941) and that they appear after many inoculations by means of infected blood indicate that exoerythrocytic stages are not all derived from sporozoites. Not until stages could be observed with regularity in birds receiving sporozoites and within a period of time short enough to exclude the development of more than one generation could such stages be thought of with some degree of assurance as stemming directly from sporozoites. In 1943 Huff, Coulston and Cantrell proposed the name "cryptozoite" for the first generation of such exoerythrocytic stages, although security restrictions prevented

the publication of our actual discovery of these forms at that time.

Claims for the discovery of such developmental forms have been made by Missiroli (1938, 1939) and by Reichenow and Mudrow (1943) for *P. relictum (praecox)*, by Kikuth and Mudrow (1939, 1940) for *P. cathemerium* and *P. gallinaceum*, by Schulemann (1940) and by Schulemann and Spies (1940) for *P. gallinaceum*. A more detailed account of these claims and their relation to our observations herein reported will be given in our discussion section. In brief, we may say at this point that although the evidence points to the probability that some of these authors have observed pre-erythrocytic stages, in none of their accounts is there presented a complete series of stages from sporozoite to erythrocytic trophozoite.

MATERIALS AND METHODS

Parasite. The strain of *P. gallinaceum* used in these experiments was strain 8A obtained through the Rockefeller Institute from Dr. E. Beltran at the Institute of Hygiene and Tropical Diseases in Mexico. This is the strain isolated by Brumpt in 1935 in Ceylon and sent by him to Beltran. It has been transmitted in this laboratory both by mosquito and blood passage since October, 1941. Because of the possible danger which could result from the escape of this parasite from the laboratory, all necessary steps were taken to prevent this from happening.

Avian Hosts. The chickens used in these experiments were white leghorns. The size of the chicken used depended on the type of experiment. Practically all wing skin injections were performed on chickens weighing not less than 1,000 grams, since it was found that such skins could be sectioned more easily than those of smaller chickens. On the other hand, small chickens, 50-100 grams, were used in all experiments where large numbers of sporozoites were injected intravenously in order to keep the dilution factor of sporozoites to blood at a minimum.

All chickens were raised in our laboratory free from ecto-parasites and there was a negligible number of animals with the common diseases of chickens. The animal house lighting was so arranged that there was approximately 12 hours of light followed by 12 hours of darkness.

Mosquito hosts. *Aedes aegypti* was used throughout these experiments. The mosquitoes were derived from a strain that has been bred in the laboratory for over three years. This mosquito is relatively easy to raise in the laboratory. Detailed accounts of the breeding, and rearing, and infecting of them will be found elsewhere. Suffice it to say that, in general, the methods of Shannon and Putnam (1934) were employed. Newly hatched mosquitoes were fed on infected birds and kept at 28°C. for 10 days. These were tested for degree of infectivity by observing the relative numbers of sporozoites in the glands of sample groups of mosquitoes. The infected *Aedes* were stored at 22° until needed. Infected mosquitoes usually had between 50 and 200 oöcysts on the midgut, some as many as 1,000. Such infections assured a plentiful supply of sporozoites and made possible the results herein reported.

Collecting and handling sporozoites. For dissection, mosquitoes were etherized lightly and the wings and legs removed. The glands were teased into a drop of equal parts chicken serum and distilled water. This mixture was used because it had been found that sporozoites would survive longer in it than in whole blood or serum, or in solutions of differing p_H and salinity. In experiments in which large concentrations of sporozoites were needed in a small inoculum, the glands were massed together and drawn into the tip of a 27 gauge hypodermic needle. This was best accomplished by observing the glands and the needle tip under a Greenough binocular microscope. Often the glands were gently teased before they were massed together, and taken into the needle. Much of the success of certain of our experiments, (i.e. wing skin) was due to this technic, and so it is described in some detail.

It is advisable to wet the plunger and barrel of the syringe with the mixture of serum and water. Since a small amount of fluid is necessary in the inoculation, the fluid in the syringe should be kept at a minimum. However, in order to make the injection practicable, it was found that leaving .01 cc of fluid in the syringe before the glands were sucked up into the needle was advantageous. This small amount of fluid behind the glands aided in forcing them out of the needle. After the glands are in the needle there should not be more than .03 cc of fluid in the syringe. Otherwise some of the glands may be drawn up into the barrel of the syringe and if this occurs, there is little likelihood that they will be forced back through the narrow opening of the syringe into the needle when the injection is made. All air bubbles should be removed since the glands and sporozoites adhere to these. Best results were obtained when

clean, new needles and syringes were used. After the inoculation, the needle was examined by forcing out any remaining fluid onto a clean slide and examining it for salivary glands. If more than half the glands were injected, the experiment was considered successful. Actually, in our experience not more than 20% of the glands were left behind in the needle when the above precautions had been followed.

Most of the cryptozoites described in this paper were from preparations of chicken skin. The method of inoculation was the one described above, and the site of inoculations was the wing web that extends across the humero-ulnar articulation. (This is the spot where wing bands are usually inserted.) Intradermal injections were made into the dorsal side of the wing skin (wing web skin). The skin on the ventral side of the web is thinner than that on the dorsal side and does not lend itself as well either to injection or to sectioning. Satisfactory inoculations were those that produced blebs resembling small blisters. These were produced by barely inserting the needle below the surface of the skin. Some deeper inoculations were made in which no bleb resulted. It was necessary that the bleb be produced in large chickens weighing over 1,000 grams in order to localize the inoculum.

One of the most important phases of this type of experiment is the marking of the area of inoculation so that it can readily be found when it is to be removed and fixed. Many methods were employed which included injections of dyes and local marking with a variety of substances. The following method was used routinely. An area between at least two or more feather follicles was chosen as the site of inoculation. Following the injection, shafts of the feathers were cut at the point where they enter the skin. When the shaft is cut, it is desirable that some bleeding or exudation of serum occur. A suspension of ordinary china ink in water is applied to the cut feather and gently worked in. This marks the area for a period of weeks and yet does not disturb the injection area. In section, it is observed that some of the china ink gets into the feather follicle and is taken up by macrophages at the bottom of the shaft. This has served as a further valuable aid, in finding the injection mass. A drawing is usually made of the bleb in relation to the feather follicles to serve as a guide when the tissue is removed.

A useful method for finding the injection area was to search for pieces of mosquito debris. These are usually found closely associated with the salivary glands and can be easily observed with a magnification of 200X. The debris consists of scales, miscellaneous bits of chitin, tracheae, etc.

Often such an area is surrounded by an intense inflammatory response which is easily recognizable. This is discussed in detail in another portion of this paper. (Fig. 46)

When other sites of tissue were injected, such as liver, spleen, muscle, kidney, bone marrow, and brain, the same method of putting the glands in the needle tip was employed. In the case of bone and skull, it was best to drill a small hole at the desired place and insert the hypodermic needle into it, rather than force through the area.

In several instances injections of sporozoites were made into areas of skin stimulated by the injection 3 days previously of salivary glands from 50 uninfected mosquitoes.

For intravenous inoculations it was necessary to grind the glands so as to obtain, as nearly as possible, an homogenous suspension of sporozoites. To accomplish this, a glass mill was used which consisted of 2 ground glass tubes, one fitting tightly within the other, the ends of which were rounded and closed. It is desirable that the upper end of the outside tube be flanged so that fluid containing sporozoites would not be spilled during the grinding. The mill is operated by hand, since it was found that when a motor was used the action was too drastic. One or 2 minutes of whirling by hand is sufficient to grind 200 sets of glands. Bits of chitin, scale, trachea, etc., which cannot be separated from the glands are usually ground into smaller pieces that easily go into a 27 gauge needle. It was necessary to grind the glands in many portions since the mill we used held only about .5 cc efficiently. After the removal of the sporozoite suspension, the mill was always washed out with equal parts of normal serum and water. Such an homogenous suspension may have as many as 15 sporozoites in a microscopic field (900X) of an air dried Giemsa stained smear. It was found that for practical purposes, the sporozoites in such a mixture were equally suspended. Only at the meniscus and in the debris at the bottom of the tube would there be a greater concentration of sporozoites, but this was a negligible factor in our experiments since all of the fluid was injected. Usually 200-400 mosquitoes glands were ground and prepared in a suspension of serum and water. This was inoculated intravenously into the leg, toe or wing vein; occasionally directly into the heart.

Many experiments were performed in which 10 to 100 mosquitoes were allowed to bite on a small circumscribed area, 2 to 20 mm in diameter. This was done by covering the bitten area with adhesive tape, having a hole, the desired size, cut into it. Often the skin was removed and the mosquitoes allowed to bite directly on the exposed muscle. This was done to facilitate the

marking of the area since the skin is easily movable whereas the muscle is firm, and also to exclude the depositing of sporozoites into the skin. Mosquitoes were also made to bite directly on the wing web skin.

Histological methods. Tissues and wet fixed smear preparations were routinely fixed in Zenker-formalin (10% neutral formalin added to 90% Zenker stock). Tissues were left in the fixative from 3 to 6 hours depending on the size and thickness of the material. Organs such as spleen, liver and brain, were sliced into small pieces not thicker than 3 mm with a sharp, thin razor. Bone marrow was fixed *in situ* following removal of part of the bone to allow penetration of the Zenker fluid.

Such tissues as skin and peritoneum were always taken in as normal a position as possible by pinning the tissue with cactus thorns to a piece of cork and then removing the area to be fixed. The cork with tissue attached was sunk in the bottle of fixative. In fixing skin the feathers were removed as much as possible since they interfered with the penetration of the fixative. However, to avoid bleeding, they were not plucked nor cut too far down the shaft. Wet fixed smears were placed in Zenker-formalin for 3 to 5 minutes, then prepared and stained in the same manner as sections.

After fixation, tissues were washed and prepared by standard methods. Following iodine treatment in 70% alcohol, the tissues were dehydrated and imbedded in celloidin. Skin was placed in dioxan for 48 hours before treatment with absolute alcohol which seemed to aid in the preparation of skins for sectioning.

Sections were routinely cut at 4 to 6 microns in thickness. Four-micron sections were especially useful in studying the detail of parasites (figs. 27-29). Often in sectioning the skins, thick sections or large pieces lifted out of the celloidin in the process. In either case, the pieces were saved and reembedded. Not infrequently a single skin had to be reembedded many times before it was cut. This applies primarily to chicken skins. Most skin sections were cut parallel to the epithelial surface. These, as well as those of other tissue injection areas, were cut serially if possible.

Staining of sections and wet-fixed smears was by the Maximow method as used extensively in this laboratory (see Huff and Bloom (1935)). Briefly this consisted of placing the sections and smears overnight in dilute Delafield's hematoxylin and counterstaining with a mixture of eosin-azure II. After clearing in xylene, sections and smears were mounted in clarite.

When an examination of stained sections or smears within a few hours was desired, a simplified technique employing Giemsa's stain followed

by dioxan and mounting in diaphane was used (for details see Coulston, 1941). Air dried smears and tissue preparations were made in the usual manner. They were rapidly dried, fixed in absolute methyl alcohol and stained with Giemsa or with Mac Neal's tetrachrome.

Cellular nomenclature. We should like to emphasize that the present study is primarily a study of the life-cycle of the parasite and not a detailed exposition of cellular reactions of the host to the parasite. However, since so much of the life-cycle of this parasite occurs in cells other than erythrocytes it is necessary to discuss briefly the types of host cells involved and their inter-relationships. Furthermore since the inflammatory response of the host to either natural or artificial injection of sporozoites may be associated with and possibly be essential to the development of the cryptozoites locally a complete understanding of the relationship between the parasites and the cells of the inoculation site is dependent upon histopathological studies not yet made.

We shall use herein the system of cell nomenclature followed by Maximow and Bloom (for details as applied especially to malaria see Huff and Bloom, 1935, and Taliaferro and Mulligan, 1937). Essentially this consists of (1) stem cells (hemocytoblasts or large tissue lymphocytes) and, (2) derivations from them consisting of small and medium lymphocytes, monocytes, and the myeloid series. Of these cells we are peculiarly concerned with the lymphoid-macrophage system a term proposed by Taliaferro and Mulligan (1937) for the macrophages (cells of reticulo-endothelial system) and their precursors. Granulocytes and true endothelial cells are involved but to a lesser extent. One important phase of the cellular activities of the host which had special significance in the studies presented here was the rapidity of the cellular response as compared with mammals. Thus some macrophages of the chicken took on the fibroblast appearance as early as 24 hours. Special attention has been called to this greater speed of cellular activities in birds by Taliaferro and Bloom (1944).

EXPERIMENTAL

Before success was attained in solving the problem of the fate of the sporozoite a number of approaches were made. These included (1) attempts to grow the sporozoites in capsules with semi-permeable membranes which were inserted in the abdomen of the canary (see Coulston (1940)), (2) attempts to grow them in cultures of chick tissues,

and (3) the inoculation of sporozoites directly into organs and the subsequent examination of those organs by means of smears and sections. Each of these methods was abandoned either because too many artifacts were introduced by the technic which might be confused with any possible early developmental stages of the sporozoite or because the dilution factor was so great as to make the finding of these stages a matter of chance. The goal that was sought was the development of a technic which not only would reveal the complete story of the growth of the parasites during the pre-patent period but would be so reliable as to make possible either the finding of parasites under a variety of conditions or to reveal their failure to grow with reasonable assurance. The method of studying these stages in maximum concentrations in a small area of wing skin has proved to possess these advantages. On the other hand, as will be shown below, there is enough evidence from other methods to show that the development of the parasites in the skin is not an abnormal one, and consequently that it can be used as a reliable index of the behavior of the parasite at times when it would be difficult to find the latter with any degree of regularity by the other methods.

For all stages of the parasite between sporozoite and erythrocytic trophozoite we shall use the term *pre-erythrocytic stages*. They are, of course, exoerythrocytic in the broadest meaning of the term. For the first generation of pre-erythrocytic stages we shall use the terms *cryptozoic generation* or *cryptozoites* (Huff, Coulston, and Cantrell, 1943). For all pre-erythrocytic stages exclusive of the cryptozoites we propose here the terms *metacryptozoites* or *metacryptozoic stages*.

Sporozoites. The morphology and staining characteristics of sporozoites

seen in dry fixed preparations stained by a Romanowsky method are well enough known to make unwarranted a redescription here. However, since practically all of our observations on the stages derived from sporozoites have been made on wet fixed preparations stained in sections (Maximow's method), a few words are necessary on the differences that exist between the appearance of the parasites in such preparations and those made by the dry fixation methods. These differences apply to most of the subsequent descriptions. They are the differences mentioned by Huff and Bloom (1935) for *P. elongatum*, by Porter (1942) for *P. cathemerium* and

P. relictum matutinum, by Huff (1942) for *Leucocytozoon simondi* and *Haemoproteus columbae*, and by Thompson and Huff (1944a) for *P. mexicanum*. In general they are the differences shown in Table 1. (See also figs. 2 and 5; 50 and 51; 54 and 55).

In our wet-fixed preparations of freshly dissected sporozoites the position occupied by the red nucleus in dry fixed smears is occupied by a nearly *achromatic vacuole* figs. 5b-f. However, within 30 minutes after their entrance into the intercellular spaces of the skin the structure which by its position should be considered as a nucleus may be an ovoid body of a blue color, darker

EXPLANATION OF PLATE 1

PLATE 1.—Sporozoites of *P. gallinaceum*, stages in the development of cryptozoites and metacryptozoites in the chicken, and unidentified artifacts.

Figures 2a-f and 3 are from air-dried smears stained by Giemsa's method. All others are from sections or wet-smears of tissues fixed in Zenker-formalin and stained by Maximow's hematoxylin, eosin-azur method (40-41, from smears; others from sections). Magnification $\times 1308$. Drawn by Esther Bohlman.

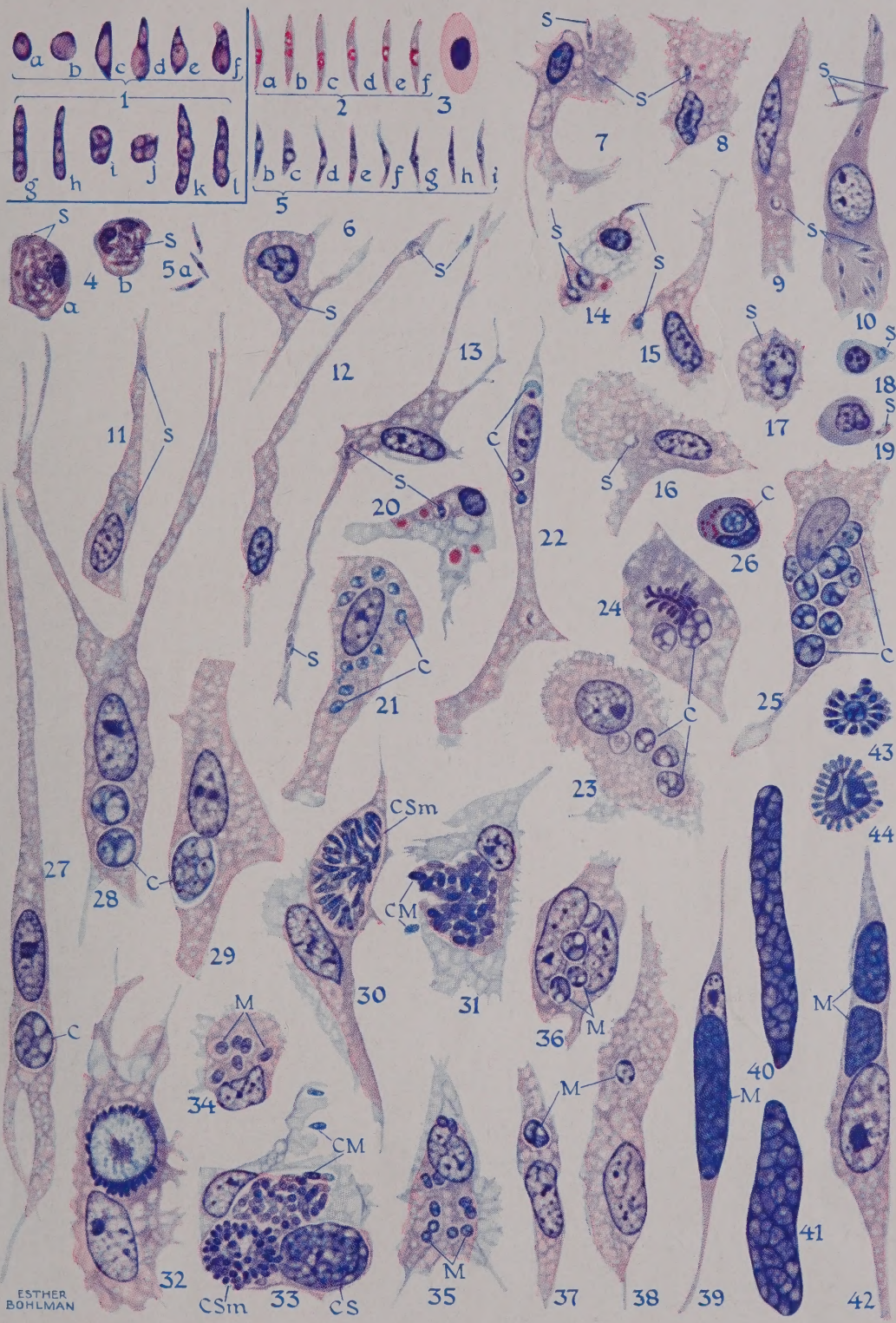
All host cells except those in figs. 4a, b and 26 are mononuclear phagocytic connective tissue cells of skin. The macrophages and fibroblasts are difficult to distinguish from each other especially the transitional cells in areas of inflammatory activity. Unless otherwise stated in the following captions it will be assumed that it was impossible to identify the host cells more accurately.

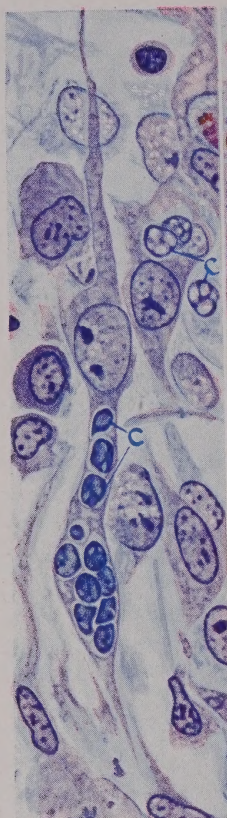
C.—cryptozoite; CM.—cryptozoic merozoite; CS.—cryptozoic schizont; CSm.—cryptozoic segmenter; M.—metacryptozoite; S.—sporozoite.

Figures 1a-1l.—Unidentified artifacts (which might be confused with cryptozoites) from injection sites in pectoral muscle.

Figures 2a-2f.—Sporozoites in air-dried smears stained with Giemsa; 3, uninfected erythrocyte; 4a, b, heterophil leucocytes (without granules) with ingested sporozoites at 6 hours; 5a, sporozoites in sections of injection area after 30 min.; 5b-i, sporozoites from wet-fixed, newly dissected salivary glands (note larger size); 6-8, 12-15, and 17-20, phagocytic connective tissue cells with inclosed, adherent, or free sporozoites (s) after 30 minutes in wing skin; 7 and 13 represent typical macrophages, whereas 18 is a lymphocyte with increased cytoplasm and 19 is a monocytoid polyblast; 14, 18, 20 are probably of lymphoid origin; 9-11, 16, cells with intracellular and extracellular sporozoites 1 hr. following injection into area previously stimulated by the injection of normal salivary glands; 16, a typical macrophage; 21, six-hour cryptozoites in a fibroblast or macrophage-transitional cell from previously stimulated area; 22, thirteen-hour cryptozoites; 23, cryptozoites, 16½ hours old; 24, two cryptozoites (16½ hours) in cell undergoing mitosis; 25, nine 24-hour cryptozoites in fibroblast; 26, probably an eosinophil leucocyte with 24-hour parasite; 27-29, probably fibroblasts with cryptozoites, 29½ hours old; note prominent nucleoli in 29; 30-35, parasites 41 hours after inoculation of sporozoites into birds recovered from sporozoite-induced infections; 30-31 cryptozoic segmenters; 32, cryptozoic segmenter with large, central clear area; 33, mature schizont and 2 segmenters in one cell.

Figures 34-35, first stages of 2nd generation (metacryptozoites); 36-38, sixty-hour metacryptozoites; note similarity to cryptozoites of figs. 23 and 24; 39, 42, fibroblasts with 70-hour metacryptozoites; 40, 41, seventy-two hour metacryptozoites from wet-fixed smears of brain; note cytological detail (host cells not shown); 43, 44, segmenters with central cores (from pectoral muscle 5 days after mosquito bites).





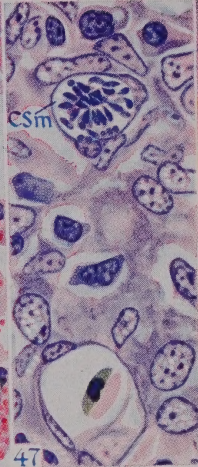
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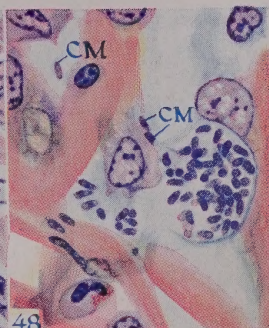
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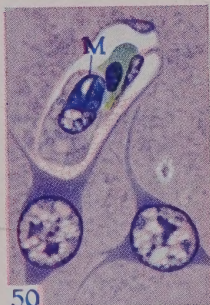
47



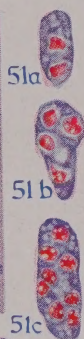
48



53



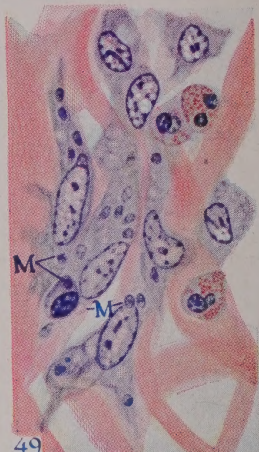
50



51a

51b

51c



49



54



55



56



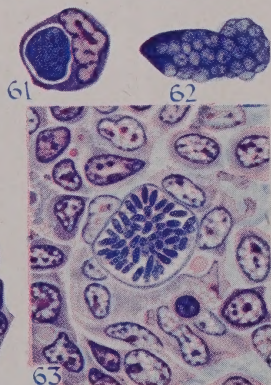
57



58



59



63



64

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than the cytoplasm (figs. 7s, 9-10s). Other sporozoites (fig. 5a) possess the darker blue body just mentioned but have conspicuous vacuoles on either side of it.

Thirty minutes after injection of the sporozoites into the skin a large proportion of them (perhaps more than 90%) lie in the intercellular spaces. Many lie superimposed on host cells in such a manner as to make difficult or impossible the determination whether they are intracellular. A few lie confined in vacuoles (figs. 7-10, 14-17) too small to accommodate the normal length of the sporozoites. Hence they assume a coiled, C-shape or a twisted, S-shape. Such obviously intracellular forms appear within the first hour in macrophages and lymphoid cells in transition to macrophages (polyblasts). Frequently

TABLE 1.—*Differences in Appearance of Malarial Parasites when Studied by Wet and by Dry Fixation Methods*

Appearance of:	By Zenker's fixation and Maximow's stain	By dry fixation, methyl alcohol, Romanowsky stain
Cytoplasm	Dark azure	Pale lavender, pink or blue.
Nucleus	Achromatic or pale blue, sometimes pale lavender; vacuole-like	Red (alizerin); variable in size and shape; often with small vacuoles.
Nucleolus	Dark purple and dot-like if present; often not visible	When present a bright red granule
General contour	Three-dimensional; little evidence of distortion due to technique in sections.	Flattened into a thin layer; evidence of severe distortion especially in nucleus.

the sporozoites appear to be trapped in the cytoplasmic processes of the macrophages (figs. 7, 10-13, 15) while in the other cells they seem to be confined to vacuoles in the central portion of the

EXPLANATION OF PLATE 2

PLATE 2.—Cryptozoites and metacryptozoites of *P. gallinaceum* in various tissues of the chicken.

Figures 51 and 54 are from air-dried smears, stained with Giemsa; all others are from sections fixed in Zenker-formalin and stained by Maximow's hematoxylin, eosin-azur method. Magnification $\times 1146$. Drawn by Esther Bohlman.

The same difficulties are encountered here as indicated in plate 1 in regard to differentiation of types of host cell.

C.—cryptozoite; CM.—cryptozoic merozoite; CS.—cryptozoic schizont; CSm.—cryptozoic segmenter; h.—heterophil leucocyte; M.—metacryptozoite; MSm.—metacryptozoic segmenter; t.—trachea.

Figure 45.—Twenty-four hour cryptozoites in fibroblasts of skin; 46, edge of injection mass in skin after 40 hours; note trachea of mosquito (t), heterophils (h), cryptozoic segmenter (CSm) and schizont (CS); 47, spleen, 36 hours after intravenous inoculation of sporozoites from 200 mosquitoes; cryptozoic segmenter probably in endothelial cell of arteriole in Schweigger-Seidel sheath; note core in segmenter; 48, cryptozoic segmenter in skin of immune animal 41 hours after sporozoite inoculation; 49, microscopic field adjacent to area of fig. 48 showing reentry of cryptozoites into a small polyblast and other mononuclear phagocytic connective tissue cells.

Figures 50-51, 54-55, sixty-hour metacryptozoites in sections and smears of brain and heart to show contrasting appearance of the parasites by the two methods; 50, brain section; 51, air-dried brain smears; 54, air-dried heart smear; 55, section showing metacryptozoite in endothelial cell of capillary of heart; 52, seventy-two hour metacryptozoite in area of spleen similar to that of fig. 47; note the arteriole; 53, 58, seventy-hour metacryptozoites in wing skin; 56, parasite in endothelial cell of heart of same animal as in fig. 52; compare parasites in figs. 55 and 56; 57 (see 64); 59-61, wing skin macrophages with 70-hour metacryptozoites; 59, hyperphagocytosis among macrophages of the skin; 62, five-day metacryptozoite in injection site of liver, note cytological detail; 63, metacryptozoite of the macromerozoite type in the edge of the arteriole sheath of the spleen 90 hours after intravenous sporozoite inoculation; on the left, note bordering lymphocytes of Schweigger-Seidel sheath; 64, pectoral muscle 5 days after mosquito bites, showing one segmenter (A) with macromerozoites and another (B) with micromerozoites; (Fig. 57, segmenter of micromerozoite type probably in endothelial cell; note clefts in parasite; from same section as fig. 64.)

cytoplasm nearer the nucleus (figs. 6, 14, 16, 17).

After 6 hours no sporozoites were seen in the intercellular spaces. At about this time, however many of them were seen inside heterophil granulocytes. These granulocytes were usually found in and around the injected salivary glands. The numbers of such inclosed sporozoites varied from a few to more than 25 (figs. 4a, b). Their cytoplasm was light lavender in color and their nuclei were somewhat darker. The leucocytes had lost their granules and could only be identified by their nuclei and by observing the series of changes which these cells undergo in the inoculated site. Since few subsequent stages of the parasites were ever seen in this cell type it must be assumed that practically all sporozoites ingested by them were killed and digested.

Cryptozoites in skin. Since a complete series of developmental forms of *P. gallinaceum* is found in macrophages from still recognizable sporozoites to mature segmenters we shall begin calling them cryptozoites as soon as they enter such cells. We, however, do not wish to restrict the term to stages which develop in this type of cells since it is conceivable that cryptozoites of this and especially of other species such as *P. elongatum* or *P. vivax* may grow in cells other than macrophages. Six hours after their entry into the bird the only cryptozoites so far seen in the skin, have been in macrophages, at which time they have assumed the oval or spheroidal shape (fig. 21). From our observations on stages intermediate between the spindle-shaped sporozoites and the rounded cryptozoites we believe the transformation from the former to the latter is a simple process of thickening in the central portion and a blunting off of the pointed ends.

From the 6th to about the 17th hour

there is little observable change in the cryptozoites aside from their growth in size (figs. 22-24). At the end of this period there are several vacuoles but usually only one, the nucleus, has the well developed spherule which we interpret to be the nucleolus. From 18 to 24 hours most of them appear to become binucleate (figs. 25 and 45) and to increase further in size but do not change in general shape. Aside from a few fairly small forms in a granulocyte (fig. 26) at 24 hours all forms were in macrophages and other mononuclear phagocytic connective tissue cells. The macrophages were either fixed or mobilized of histogenous and hematogenous origins. Many of them had already changed in appearance to resemble fibroblasts (figs. 27, 28, 29,). Some of the infected cells contain large numbers of parasites, while others have few or single ones.

From 24 to 36 hours after inoculation the cryptozoites grow rapidly in size while retaining their shape which, in some, is nearly spherical (fig. 28) and in others is that of a prolate spheroid (fig. 29) or occasionally egg shaped (fig. 27). The number of structures which appear to us to be nuclei increase in number from 2 to over 100. In sections from the same piece of skin taken at this time, cryptozoites have been seen having as few as 4 nuclei or as many as 24. The large number of vacuole-like nuclei in the larger schizont give the latter a bubbly appearance (fig. 29, 33cs). The pre-segmenters appear to consist of a densely stained inner core on the outside of which there is a layer of bubble-like vacuoles closely crowded together and giving a foamy appearance. The first mature segmenters were seen after 36 hours, the majority of them were seen at about 42 hours but they continued to appear in preparations made as late as 48 hours after inoculation. In some segmenters the central

mass of densely staining material is still present in an unsegmented condition, and a peripheral layer of elongate merozoites (figs. 46, 47) incloses the unsegmented portion. The merozoites have one end resting on the inner core and the other end pointing peripherally in a manner suggesting the production of sporozoites in the oöcyst (see figs. 43, 44 for similar appearance in metacryptozoites). The individual, cryptozoic merozoite resembles a sporozoite except that it is shorter and the details of its inner structure are somewhat more difficult to make out than that of the sporozoite. In other segmenters the merozoites are haphazardly arranged and the central core may be missing or replaced by a conspicuous, clear area (fig. 32). We do not know what happens to the unsegmented core but we think it probable that a second crop of merozoites may be formed from it. This view is supported by the fact that both in the cryptozoic segmenters and the metacryptozoic segmenters (to be described below) the earliest segmenters contain the solid central core whereas the ones seen later in point of time but within the same period of segmentation usually contained a central, clear area instead of the unsegmented core (fig. 32). One macrophage may contain more than one large schizont or segmenter. Fig. 33 shows such a cell containing a large schizont, a mature segmenter and a segmenter in the process of bursting out of the host cell.

That many of the cryptozoic merozoites after leaving the host cell in which they were formed enter adjacent macrophages is clearly shown by our preparations where there are small areas in which most of the macrophages are infected (figs. 48, 49). There is evidence which we shall later present indicating that some of the cryptozoic merozoites migrate to other positions in the host

and invade macrophages or other cells such as endothelial cells which were seen to be infected after 60 hours.

Cryptozoites in the spleen. In addition to the cryptozoites described above from the inoculation site in the skin we were able to find them in the spleen of animals which had been inoculated with massive doses of sporozoites. The salivary glands from 200–300 infected *Aedes aegypti* were ground and inoculated intravenously into each of many small chicks. The chicks were sacrificed at varying intervals of time and the organs fixed, sectioned and stained. In the spleen of a few of these we found large schizonts and segmenters usually completely filling the small arterioles (fig. 47) and capillaries of the Schweigger-Seidel sheaths (fig. 63). The cells in these sheaths are feebly phagocytic and may be reticular cells (Taliaferro, 1944). Often it was thought these cryptozoites were in endothelial cells lining these small vessels. Since their manner and rate of growth was the same in the spleen as in the skin we must assume that the development of the sporozoites can normally occur in either location. Although a thorough study of the localization of cryptozoites has not yet been made it is probable that they may occur in sites other than the spleen as, indeed, the subsequent stages do.

Metacryptozoites. The generations which follow the cryptozoites are, with some exceptions, practically indistinguishable from the latter. We have as yet no evidence that cryptozoic merozoites enter erythrocytes. Reentry of macrophages and other mononuclear phagocytic connective tissue cells by the cryptozoic merozoites has been seen to occur in skin as early as 41 hours (figs. 34, 35, 48, 49). At that stage the parasites resemble closely those of the first generation as they appear at about 5 or 6 hours. At 60 hours they are indis-

tinguishable morphologically from cryptozoites about 24 hours old, except that some of them are more advanced in development (figs. 36-38). The same differences in staining from the dry smear and the wet fixation smear or section technics as mentioned above for the sporozoites and cryptozoites are observed in these forms (Cf. figs. 50 and 51; also 54 and 55). The parasites in the inoculation site are much more numerous in this generation than in the cryptozoic one.

The development from 60 hours to 72 hours is very comparable to that which occurs between 24 and 36 hours (figs. 39-42, 52-53, 56, 58-61). Multiple infections of cells of the lymphoid-macrophage system are very common (fig. 53). In a few birds we have seen also in the local injection site at 70 hours numerous examples of a curious cannibalism amongst the infected polyblasts and macrophages. This may be complex as shown in figure 59, or simple. A large macrophage is here shown to contain 1 schizont, 1 macrophage with ingested schizont and 2 macrophages, one inside the other, and each containing a schizont within itself. The second schizogony begins at about 70 hours (fig. 58). Morphologically the large schizonts and segmenters appear to be identical with those of the first generation. Although the preponderance of merozoites from the second generation again enter macrophages and endothelial cells, we have evidence, as shown below, that they may enter erythrocytes as early as 75 hours after the inoculation of sporozoites. A complete analysis of the events taking place henceforth in our material becomes very difficult because the synchronism of growth diminishes until practically all stages of growth are encountered at a particular time and the genealogy of the various cryptozoic,

metacryptozoic, and erythrocytic stages cannot be clearly traced.

However, between the 3rd and 5th days of the infection the exoerythrocytic stages increase greatly in numbers and become more widely spread in the body, for at the end of this time practically all organs are invaded. As early as the 5th day 2 different types of segmenters are present in the local injection sites (figs. 64). Some of them are similar to cryptozoic segmenters in possessing smaller numbers of merozoites (100-200) and larger individual merozoites than do the other type (fig. 64a). Others of them resemble the exoerythrocytic segmenters which prevail in infections produced by inoculations of infected blood and also later in infections produced from sporozoites. They have very large numbers (about 500 to 1,000) of smaller, more tightly packed merozoites which also appear to stain more deeply than the cryptozoic type (fig. 64B).

In contrast to the scarcity of cryptozoites in the organs of animals inoculated intravenously with large doses of sporozoites, metacryptozoites were found to be abundant in spleen, cardiac muscle, kidney, brain, liver, lamina propria, bone marrow, thymus, etc., at the same time intervals as they were found in the skin. In these sites they were found especially in the endothelial cells and occasionally in cells of the lymphoid-macrophage system.

Since the subsequent events in the infection fall outside the scope of this paper we shall not attempt their analysis here. We shall, however, give a few facts about the early parts of the erythrocytic infections which bear upon the problem.

The beginning of the parasitemia. The earliest infected erythrocyte to be encountered was found in a small chick 75 hours after the ground salivary

glands from 300 infected *Aedes aegypti* had been intravenously inoculated into it. Following heavy inoculations (100 to 300 pairs of infected salivary glands) blood parasites are found after long searching of smears taken at 90 hours and fairly regularly in smears taken after $4\frac{1}{2}$ or 5 days. With moderate inoculations the prepatent period extends to 6 or 8 days. Following the bite of one or a few infected mosquitoes the incubation period is 7 to 10 or more days. Following the intravenous inoculations of sporozoites there is, within a 24 hour period, at about the 6th to 8th days of infection a sudden increase of young erythrocytic trophozoites of the order of approximately 200 times the number in the preceding 24 hour period. This so-called "flooding effect" represents the climax of the change from pre-erythrocytic to erythrocytic parasites. There follows a heavy parasitism of the blood which is, however, accompanied in this species by abundant exoerythrocytic stages in the tissues. In smears or sections of brains taken at this time it is possible to observe large segmenting forms producing micromerozoites. As the merozoites are liberated they can be observed in the blood vessel on or in adjacent erythrocytes. A short distance away erythrocytes are not parasitized.

Artifacts. The inoculation of sporozoites into tissues either naturally by mosquitoes or experimentally by hypodermic needle is accompanied by degenerative changes and intense inflammatory response in the tissue which is likely to yield artifacts and objects of confusion in the search for a new stage in the life cycle of a parasite. This applies especially to the early hours following inoculation when the stages to be sought are small. The only microorganisms encountered besides the malarial parasites were (1) bacteria which

were seen only a few times inside the injection mass and (2) on one occasion a mold which was easily identified as such. Before all stages of the cryptozoites were discovered, however, numerous false leads were encountered in the form of both extracellular and intracellular globules, granules and amorphous bodies many of which we were unable to identify. One series of such artifacts proved to be particularly misleading until later findings demonstrated that it was entirely unconnected with the malarial life cycle. They were found in pectoral muscle following the injection of salivary glands from mosquitoes. A series of these artifacts is shown in figures 1a-1l. They were characterized by the very deep purple color and by the presence of vacuoles resembling the nuclei of exoerythrocytic schizonts of malarial parasites. The vacuole (which at first was interpreted as a nucleus) was single and simple or a series of smaller vacuoles were present in the dark staining area around the larger vacuole. Other forms were found which had more than one vacuole and which were believed at the time to be multinucleate malarial cryptozoites. Only after we had found the true, large schizonts and segmenters of the cryptozoites and worked backward in the series did we discover that the dark bodies were artifacts. We subsequently found them in areas into which uninfected salivary glands had been injected. We now believe it likely that some of these bodies are the degenerating nuclei of heterophil leucocytes, for we have seen a complete series connecting them with the intact granulocyte. Others may be degenerating erythrocyte nuclei.

DISCUSSION

Relation of our findings to previous work. Beginning in 1933 Missiroli pub-

lished a series of observations upon the behavior of sporozoites of *P. relictum* in the canary (1933, '34, '37, '38, '39, '41). In brief his claims were that at the site of the inoculation the sporozoites lose their cytoplasm after 10 minutes, the nuclear bodies remain normal in appearance and some of them attach themselves to erythrocytes. He postulated that the sporozoite is really a sporocyst which must undergo further changes before producing sporozoites. He observed 2, 4, 6, and 8 granules in the nuclei after a few minutes and assumed that these changes had something to do with the transformation of the sporocysts to sporozoites. After 3 hours, only degenerate forms remained. Since he had no success in finding any developing forms in muscles (inoculation site), spleen, liver, lung or bone marrow and since he believed that his amputation experiments proved that there was a rapid migration of the sporozoites from the inoculation site, he concluded that sporozoites could not complete their development at the local inoculation site. This is contrary to our findings as described later in this discussion. In 1939 he still claimed that sporozoites enter neither erythrocytes nor reticulo-endothelial cells but that they lie in the intercellular spaces and lose their cytoplasm. He later (1941) amplified these views and claimed that between 12 and 30 hours 4-nucleated schizonts develop and divide into 4 merozoites which then enter erythrocytes. We find no support in our findings for Missiroli's ideas about the sporocystic nature of the sporozoite, the loss of its cytoplasm, nor their development extracellularly. It is likely that his methods were not suited to finding the cryptozoites so abundantly found in our preparations in the local inoculation site. The appearance of the nuclei of normal sporozoites in ordinary dry smears is highly variable and we

therefore believe that Missiroli has attached undue importance to the granules which he saw in the sporozoites shortly after their entrance into the host (See our figures 2a-2d). Kikuth and Mudrow (1941, '42) have already disputed Missiroli's claims.

Kikuth and Mudrow (1939, '40) studied the inoculation sites and internal organs of canaries inoculated with sporozoites of *P. cathemerium*. They described and figured extracellular and intracellular forms from touch preparations of pectoral muscle 16 hours after sporozoite inoculation. These bodies as seen in air dried preparations were predominantly uninucleate, with dark blue cytoplasm and light red nuclei. They were uncertain of the nature of the infected host cell but thought that it was probably monocyte-like. They found a total of 36 parasites in preparations made after 40 hours. These forms were chiefly intracellular and some infections were multiple. These parasites again were usually uninucleate and, on basis of our findings, probably belonged to the second generation. After 48 hours many of the stages found in pectoral muscle were multinucleate, a 5-nucleated one being the latest stage found. They saw sparse multinucleate stages after 64 hours. Although the technics used by Kikuth and Mudrow were so different from ours that a comparison of the figures from the 2 methods is not conclusive, we believe the evidence is good that they did see cryptozoites and metacryptozoites of *P. cathemerium*. Due to the fact that most of their studies were made on pectoral muscle in which tissue we found the greatest number of artifacts and that no large schizonts or segmenters were found, there is some question that some of the structures described by them may have been artifacts similar to the ones described here by us (figs 1a to 1l). If their preparations actually

contained cryptozoites the time intervals were such as to miss many of the most interesting stages. Their statement that "through our findings the events in the development of *P. cathemerium* from the penetration of sporozoites into the bird until the appearance of the first erythrocytic parasites are essentially clarified" seems somewhat premature in view of their very incomplete findings.

Schulemann and Spies (1940) implanted pieces of sterile sponge under the skin of chickens to bring out aggregations of "histiocytes." Eight to 10 days later a suspension of sporozoites of *P. gallinaceum* in equal parts of chicken serum and 0.9% saline was injected into the area. This was preceded by an injection of either dianil-violet or colloidal palladium to mark the histiocytes. In sections of preparations taken 24 hours after the sporozoites were injected 4-nucleate stages, both intra- and extracellular, were found. After 40 to 65 hours larger forms with many nuclei were also found. The intracellular forms were in cells which also included granules of dianil-violet or palladium. Hence on a functional basis, Schulemann and Spies concluded that the pre-erythrocytic stages grow in histiocytes. No mention was made of segmenting forms and we must assume that they were not seen. Contrary to our findings, they claimed that sporozoites were present in the tissues up to 4 days. The chief contribution of this work was the evidence concerning the nature of the infected host cells. No figures were given, however, so it is difficult to determine whether they actually saw the cryptozoites. Schulemann (1942) also worked with *P. cathemerium* but the main result of his work with that species was a claim that the sporozoite undergoes a division into 2 parts as described by Missiroli. As indicated above, we

are not in agreement with that view.

In a paper by Reichenow and Mudrow (1943)* the early development of *P. praecox* (= *relictum*) in canaries is described. Many of their claims agree with our findings (1942) on *P. gallinaceum*. They inoculated the birds intramuscularly with infected salivary glands which had been ground in physiological saline. Observations were made from 13½ to 189 hours following inoculations. Most of the forms they saw during the first 24 hours were uninuclear. At 35 hours they found 2 schizonts with 4 nuclei, 2 with 16 nuclei and 1 with 24 nuclei. At 40½ hours they found 1 "ripe schizont" which we interpret as meaning a segmenter. The first infected erythrocytes were found at 115 hours. They believed the infected cells were the "reticulo-endothelium and macrophages" and found them most abundantly "in advanced infections" in the spleen and liver. They confirmed our findings (1942) on the presence of two types of segmenters and named the merozoites derived from them "macromerozoites" and "micromerozoites." On the basis of finding one segmenter they estimated the length of the first generation to be approximately 36 hours. They of course missed the early transformation of sporozoites into cryptozoites which we have shown to occur in the first 6 hours.

This is not the place to compare our work in detail with all of the findings of Kikuth and Mudrow or of Reichenow and Mudrow upon *P. cathemerium* and *P. praecox* (= *relictum*) since we have done extensive investigations upon both of these species also and our results will appear in subsequent papers. However, we may conclude that although their work, is very important in giving frag-

* This paper was made available through the courtesy of Dr. G. Robert Coatney.

mentary knowledge of the life-cycles of the respective species, in no case yet known to us has a complete cycle from sporozoite to erythrocytic trophozoite been described.

Technics. Our development of a method by which maximum numbers of sporozoites are injected into an area of tissue in minimum amounts of fluid and by which this tissue may be fixed, sectioned, and stained so as to study the sporozoites and their descendants as well as the exact histological details of the tissue itself, presents nothing in itself novel or revolutionary. The perfection of the method to make of it an effective tool in studying not only the life cycle of the malarial organisms themselves but their behavior under a variety of conditions such as the natural and acquired immunity and experimental therapeutics of the host does, perhaps, merit some emphasis. Much of the experimental work done in parasitology leaves unknown the important events which transpire between the moment of inoculation and the appearance several generations later of the parasites in the blood, excreta, or tissues in numbers large enough to make their study practicable. Perhaps, in no parasitic disease is this unknown period of development more important than it is in malaria. Our results with the method on immunity and action of antimalarial drugs, some of which will soon be published, make us feel confident that either this method or modifications of it can be used in securing the answer to many problems in malariology.

Cytological observations. The peculiarity of the staining of malarial parasites by the methods generally used for vertebrate tissues has been noted previously (Huff and Bloom, 1935; Porter, 1942; Huff, 1942; Thompson and Huff, 1944a). The appearance of the structure which by its location and its appearance under

dry smear technics, would be called nucleus is so different from the nuclear material of animals in general as to cast doubt upon the chemical similarity of the two substances. Furthermore although many hundreds of schizonts have been studied in all stages of growth no evidence has been seen of mitotic behavior. This has been especially noted previously in *Leucocytozoon simondi* (Huff, 1942).

The method of merozoite formation, especially in the first few generations of pre-erythrocytic segmenters is different from anything yet observed in malarial organisms unless we include some of the happenings in the sporogonous development of *Plasmodium* in the mosquito. As noted above the appearance of the unsegmented core surrounded by a peripheral layer of merozoites occurs more frequently toward the beginning of the segmentation period whereas the segmenters seen later in the same segmentation period have the merozoites peripherally arranged but a large vacuole replaces the unsegmented core in the interior. This not only suggests the possibility that merozoites may be formed in successive crops but that these different sets of merozoites may be genetically differently constituted and may therefore have different potentialities.

The transition period. Between the first 2 generations of tissue stages of *P. gallinaceum* on one hand and the beginning of the patent infection of the blood on the other hand there is a period of transition during which the parasite changes from complete tissue parasitism to parasitism characterized by its localization in the blood. During this period there begins the parasitism of the blood accompanied by infection of the lymphoid-macrophage system and endothelial cells with metacryptozoites, and also by the appearance of two morphologically different types of exoerythro-

cytic segmenters. Due to the overlapping of these phenomena it is difficult to prove unequivocally the genealogies of the various stages and the degree to which they change from one kind to another.

Without claiming that we can present enough evidence to prove the matter unequivocally we believe the following hypothesis to accord best with the facts. The parasitism of erythrocytes which begins at least by the end of the second generation of pre-erythrocytic stages increases in amount with each successive segmentation of pre-erythrocytic parasites. If this be a logarithmic increase the steep portion of the graph of their numbers would then correspond to the "flooding effect" mentioned earlier. This flooding effect is the sudden increase in the blood of numbers of erythrocytic trophozoites many times that which could be accounted for by the multiplication of erythrocytic trophozoites through schizogony. At some time after the cryptozoic generation 2 types of segmenters appear which form *macromerozoites* and *micromerozoites* (to use Reichenow and Mudrow's terms). There is then a shift in successive generations from the former to the latter type of segmenters and merozoites. A similar difference in types of segmenters has been observed in various species of *Eimeria* in the rat by Roudabush (1937) and in the rabbit by Rutherford (1943). Porter (1942) has already shown that there is in the tissue distribution of exoerythrocytic stages in infections induced by intravenous inoculations of sporozoites of *P. cathemerium* a shift from the parasitism of macrophages early in the infection over to a predominance of exoerythrocytic forms in the endothelial cells later. It is conceivable that there is some relationship between the shift in types of merozoites produced and the localizations in host cells in the

early stages as compared with the later stages of the infection.

The question may legitimately be asked whether cryptozoic merozoites may not occasionally enter erythrocytes. We shall not deny the possibility. However, on basis of the amount of time required for us to find one erythrocytic parasite at the end of the second generation, we have estimated that it might take several hundred man hours of searching to reveal the existence of a single erythrocytic parasite at the end of the first generation in a comparable experiment.

Development of sporozoites naturally and artificially inoculated. Since a large part of the results described herein were obtained by the injection of sporozoites by syringe instead of by the mosquito it is necessary to correlate these findings with the happenings following natural transmission of sporozoites. As mentioned in the section "Experimental" the rate of development of the cryptozoites was the same whether the sporozoites were inoculated intradermally, subcutaneously or intravenously. In experiments here reported and others to be reported later it was found that following mosquito bites sporozoites may develop at the local site of biting or may gain entrance to the circulatory system and develop elsewhere (i.e. spleen, heart, kidney). Therefore, we believe that the results obtained from the injection of sporozoites by artificial means are not essentially different from the natural course of events following bites of infected mosquitoes.

Significance of pre-erythrocytic development. The only species of malarial parasites in which cryptozoites have been found happen to possess abundant exoerythrocytic stages which live either in the lymphoid-macrophage system or in endothelial cells. The danger of assuming that all species of *Plasmodium*

necessarily go through early stages of growth in these cells is obvious. Many species either do not possess exoerythrocytic stages or the latter have not yet been unequivocally demonstrated in them. In at least one species (*elongatum*), exoerythrocytic stages exist but they occur predominantly in the erythroblastic series of cells. We believe it likely, therefore, that some species of *Plasmodium* undergo their cryptozoic development in different kinds of cells from those utilized by *P. gallinaceum*. We do not think that the possibility of a direct development from sporozoite to erythrocytic trophozoite in some species should be denied. Much indirect evidence, however, is against such a hypothesis. Surely with the great differences which are known to exist among species of malarial parasites in regard to their ability for living in different cell types of the host it ought not to be surprising to find that they also differ widely in the cell preferences shown by their cryptozoites.

SUMMARY

A technic for the study of the early development of malarial parasites in the vertebrate has been perfected.

By the use of these methods the following results have been obtained:

1. Sporozoites of *P. gallinaceum* entered cells of the lymphoid-macrophage system within 30 minutes after inoculation into the skin of chickens.

2. These cells served as hosts for all stages of the first generation of parasites (cryptozoites) from forms still recognizable as sporozoites to large schizonts and segmenters.

3. Sporozoites were found in heterophil leucocytes as late as six hours following inoculation. Only a few of these showed any appreciable development after 24 hours and none was found to

complete its development in this type of cell.

4. Sporozoites were not found in the intercellular spaces after 6 hours.

5. The first, or cryptozoic generation required about 42 (range of 36 to 48) hours whether the parasites grew locally at the site of inoculation or in the spleen.

6. In morphology and staining reactions these cryptozoites showed a resemblance but were not identical with the gallinaceum-type of exoerythrocytic stages previously known.

7. The second generation (first generation of metacryptozoites) was found in cells of the lymphoid-macrophage system and in endothelial cells. This generation underwent segmentation at 70 to 84 hours. These parasites were, in general, very similar to the cryptozoites.

8. The first erythrocytic parasite was found 75 hours after a massive intravenous inoculation of sporozoites.

9. After 60 hours numerous metacryptozoites were observed, especially in the inoculation site of the skin following intradermal inoculation and in the spleen, heart, kidney, and brain (in the order named) following intravenous inoculation of sporozoites. At the same time sparse erythrocytic parasites were seen at 90 hours after which they gradually increased in numbers up to about 6 days. Then followed a precipitous increase in erythrocytic parasites ("flooding" effect).

10. Five days following inoculation of sporozoites or the bites of mosquitoes 2 types of segmenters were abundant. One of these consisted of smaller numbers of large-sized merozoites of the cryptozoic type) macromerozoites) while the other consisted of large numbers of smaller-sized, tightly packed merozoites of the type seen in blood-induced infections (micromerozoites).

While the results of observations on

this species of malarial parasites clearly indicate that the parasitism of the blood must be preceded by a tissue parasitism it is considered to be unwarranted to assume, as yet, that such is the case with all malarial parasites. Furthermore, the kinds of tissue parasitism might possibly be very different in the various species of parasites.

REFERENCES

- Ben-Harel, S. 1923, *Am. J. Hyg.* **3**: 652-685.
- Boyd, M. F., and Matthews, C. B. 1939, *Am. J. trop. med.* **19**: 69-71.
- Boyd, M. F., and Stratman-Thomas, W. K. 1934, *Am. J. Hyg.* **20**: 488-495.
- Ciucca, M., Ballif, L., Chelaresco, M., Isanos, M., & Glaser, L. 1937, *Riv. di Malariol* **16**: 85-90.
- Coulston, F. 1939, M. A. Thesis, Syracuse (N. Y.) Univ.; 1940, *Jour. Parasit.* **26**: 30; 1941, *Jour. Lab. & Clin. Med.* **26**: 869-873.
- Coulston, F., and Manwell, R. D. 1941, *Am. J. Hyg.* **34**: 119-125.
- Corradetti, A. 1938, *Riv. di parassit* **2**: 23-38.
- Danilewsky, B. 1885, *Biol. Centralb.* **5**: 529-537.
- DeCourt, P., and Schneider, J. 1938, *Bull. Soc. Path. Exot.* **31**: 609-614.
- Grassi, B. 1899, *Atti Reale Accad. Naz. Lincei* **3**: 299-302; 1901, *Die Malaria*. Jena 242 pp.
- Grassi, B., Bignami, A. and Bastianelli, G. 1899, *Atti R. Accad. Lincei, Rendic.* **8**: 21-33.
- Henry, C. 1939, *Bull. Soc. Path. Exot.* **32**: 30-34.
- Huff, C. G. 1930, *Am. J. Hyg.* **11**: 385-391; 1942, *J. Inf. Dis.* **71**: 18-32.
- Huff, C. G., and Bloom, Wm. 1935, *J. Inf. Dis.* **57**: 315-336.
- Huff, C. G., and Coulston, F. 1942, 1944. Unpublished observations.
- Huff, C. G., Coulston, F., and Cantrell, W. 1943, *Science* **97**: 286.
- James, S. P. 1931, *Trans. Roy. Soc. Trop. Med. & Hyg.* **24**: 477-538.
- James, S. P., and Tate, P. 1937, *Nature* **139**: 545; 1938, *Parasit.* **30**: 128-138.
- Kikuth, W., and Giovannola, A. 1933, *Riv. di Malariol* **12**: 657-674.
- Kikuth, W., and Mudrow, L. 1937, *Klin. Wochschr.* **16**: 1690-1691; 1938a, *Riv. di Malariol* **17**: 1-14; 1938b, *Zentr. f. Bakt.* **142**: 113-132; 1939, *Zentr. f. Bakt.* **145**: 81-88; 1940, *Riv. di Malariol.* **19**: 1-15; 1941, *Zent. f. Bakt.* **147**: 284-288; 1942, *Zent. f. Bakt.* **149**: 98-101.
- Laveran, A. 1880, *Bull. Acad. Méd.* **9**: 1235, 1268, 1346.
- Manwell, R. D., and Goldstein, F. 1939a, *Science* **89**: 131-132; 1939b, *Am. J. trop. med.* **19**: 279-295.
- Missiroli, A. 1933, *Riv. di Malariol.* **12**: 585-586; 1934, *Riv. di Malariol.* **13**: 539-552; 1937, *Riv. di Malariol.* **16**: 99-107; 1938, *Riv. di Parassit.* **2**: 39-43; 1939, *Riv. di Parassit.* **3**: 339-342; 1941, *Zent. f. Bakt.* **146**: 353-359.
- Porter, R. J. 1942, *J. Inf. Dis.* **71**: 1-17.
- Porter, R. J., and Huff, C. G. 1940, *Amer. J. trop. med.* **20**: 869-888.
- Raffaele, G. 1934a, *Riv. di Malariol.* **13**: 332-337; 1934b, *Riv. di Malariol.* **13**: 395-403; 1936a, *Riv. di Malariol.* **15**: 77-87; 1936b, *Riv. di Malariol.* **15**: 318-324; 1937, *Riv. di Malariol.* **16**: 185-198.
- Reichenow, E., and Mudrow, L. 1943, *Deutsch. tropenmed. Zeit.* **47**: 289-299.
- Ross, R. 1898, *Indian Med. Gaz.* **33**: 401-448.
- Roudabush, R. L. 1937, *Iowa State Col. J. of Sc.* **11**: 135-163.
- Russell, P. F., and Nono, A. M. 1932, *Phil. J. Sci.* **49**: 595-625.
- Rutherford, R. L. 1943, *J. Parasit.* **29**: 10-32.
- Schaudinn, F. 1902, *Arb. K. Gesundh.* **19**: 169-250.
- Schulemann, W. 1942, *Deutsch Med. Woch.* **68**: 374-375.
- Schulemann, W., and Spies, K. 1940, *Deutsch Med. Woch.* **66**: 404-405.
- Sergeant, Et., and Sergeant, Edm. 1922, *Arch. Inst. Past. l'Afr. du Nord* **2**: 320-329.
- Shannon, R. C., and Putnam, P. 1934, *Proc. Entom. Soc. Washington* **36**: 185-242.
- Swellengrebel, N. H., and de Buck, A. 1931, *Proc. Koninkl. Akad. Wetensch. Amsterd.* **34**: 183-185.
- Taliaferro, W. H. 1944, Personal Communication.
- Taliaferro, W. H., and Bloom, W. 1944, Unpublished manuscript.
- Taliaferro, W. H., and Mulligan, H. W., 1937, *Indian Med. Res. Mem.* **29**: 1-138.
- Thompson, P. E., and Huff, C. G. 1944a, *J. Inf. Dis.* **74**: 48-67. 1944b, *J. Inf. Dis.* **74**: 68-79.
- Verney, L. 1938, *Ann d'Ig.* **48**: 26-40.
- Warren, A. J., and Coggeshall, L. T. 1937, *Am. J. Hyg.* **26**: 1-10.
- Yorke, W. 1931, *Trans. Roy. Soc. trop. Med. & Hyg.* **24**: 478-538 (see p. 527).
- Yorke, W., and MacFie, J. W. S. 1924, *Trans. Roy. Soc. trop. Med. & Hyg.* **18**: 13-33.



